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Evaluation of a Fluorescence In Situ Hybridization Assay for Differentiation between Tuberculous and Nontuberculous *Mycobacterium* Species in Smears of Lowenstein-Jensen and Mycobacteria Growth Indicator Tube Cultures Using Peptide Nucleic Acid Probes

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A new fluorescence in situ hybridization assay based on peptide nucleic acid probes (MTB and NTM probes targeting tuberculous and nontuberculous species, respectively) for the identification of *Mycobacterium tuberculosis* complex and differentiation between tuberculous and nontuberculous mycobacteria (NTM) was evaluated using Lowenstein-Jensen (LJ) solid cultures from 100 consecutive sputum samples and 50 acid-fast bacillus (AFB)-positive sputum samples as well as Mycobacteria Growth Indicator Tube (MGIT) liquid cultures from 80 AFB-positive sputum samples. *Mycobacterium* species could be identified from a total of 53 LJ cultures and 77 MGIT cultures. The diagnostic specificities of the MTB and NTM probes were 100% for both cultures. The diagnostic sensitivities of the MTB probe for the LJ and MGIT cultures were 98 and 99%, respectively, whereas the sensitivities of the NTM probe were 57 and 100%, respectively. The relatively low sensitivity of the NTM probe was due to a high proportion of *M. fortuitum*, which is not identified by the probe.

The definitive diagnosis of tuberculosis depends on the isolation and identification of species of the *Mycobacterium tuberculosis* complex (MTBC). The conventional microscopic examination of acid-fast bacillus (AFB) staining, culturing on solid medium, and a series of biochemical tests are still the methods of choice in most mycobacteriological laboratories. In order to reduce the time needed for and improve the sensitivity and specificity of identification methods, a combination of liquid media and DNA probe-based identification methods has been recommended (19, 31), and alternative identification methods have been or are in the process of being developed.

One of the new liquid medium-based tests is the nonradiometric Mycobacteria Growth Indicator Tube (MGIT; BBL Microbiology Systems, Cockeysville, Md.), which is based on oxygen-quenching fluorescent sensor technology that can shorten the recovery time for mycobacteria to as little as 1 to 2 weeks (14, 23). Among the new identification methods are high-performance liquid chromatography (3, 4), DNA sequencing (8, 16, 24, 28), PCR amplification of species-specific sequences (6, 21), amplification and restriction enzyme analysis (2, 17, 25, 29, 30), and hybridization with species-specific DNA probes (1, 5,12, 25).

Recently, a novel fluorescence in situ hybridization (FISH) test, which uses peptide nucleic acid (PNA) probes for the identification of mycobacteria from cultures, has been developed (27) and evaluated for use with MB/BacT (Organon Teknika, Durham, N.C.) and BACTEC 12B (BBL Microbiology Systems) cultures (7, 22). Further, the applicability of the method for direct use with sputum samples has been evaluated (26). PNA is a novel DNA mimic in which the sugar-phosphate backbone of DNA has been replaced with a polyamide backbone (9, 10, 20). The uncharged nature and the high conformational flexibility of PNA allow PNA probes to hybridize to DNA or RNA with excellent affinity and specificity (11). The aim of this study was to evaluate the DAKO Probe MTB Culture Confirmation Test (DAKO, Glostrup, Denmark) for the identification of species of MTBC and differentiation between tuberculous and nontuberculous mycobacteria (NTM). The PNA probe test, which utilizes two fluorescein-labeled PNA probes, the MTB probe targeting MTBC species and the NTM probe targeting NTM species, was evaluated with Lowenstein-Jensen (LJ; Difco, Detroit, Mich.) and MGIT cultures from sputum specimens.

MATERIALS AND METHODS

***Mycobacterium* reference strains.** The *Mycobacterium* reference strains *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. bovis* BCG ATCC 35734, *M. avium* ATCC 25291, *M. intracellulare* ATCC 13950, *M. scrofulaceum* ATCC 19981, *M. gordonae* ATCC 14470, *M. kansasii* ATCC 12478, *M. xenopi* ATCC 19250, *M. fortuitum* ATCC 6841, and *M. chelonae* ATCC 35752 were grown on LJ medium and tested.

Contaminating bacteria. Ten isolates of bacteria isolated from mycobacterial cultures as contaminants were grown on blood agar media. The bacteria were identified as belonging to the genus *Pseudomonas*, *Staphylococcus*, or *Streptococcus*.

Clinical specimens. A total of 100 consecutive sputum samples and 50 AFB-positive sputum samples were included in the study with LJ cultures; from these cultures, 53 *Mycobacterium* strains could be identified. A total of 80 AFB-positive sputum samples were included in the study with MGIT cultures; 77 *Mycobacterium* strains were identified. The LJ and MGIT studies were conducted separately using different specimens.

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Specimen processing. The sputum specimens were digested and decontaminated by the *N*-acetyl-L-cysteine(NALC)–NaOH method (15), with the following modification: the final concentrations of NaOH were 1.25 and 2.0% for the LJ cultures and the MGIT cultures, respectively. For both cultures, an amount of HCl equimolar to that of NaOH was added after 15 min of incubation in order to neutralize the NaOH. The digested specimens were inoculated onto the LJ and MGIT media as recommended by the manufacturers. Contamination rates for LJ and MGIT cultures were 20 and 5%, respectively.

PNA probes. The DAKO Probe MTB Culture Confirmation Test contains two fluorescein-labeled PNA probes, an MTB probe targeting MTBC species and an NTM probe targeting the majority of the clinically relevant NTM species. Both probes target mycobacterial rRNA molecules (27).

Smear preparation. For LJ cultures, a loop of colonies was suspended in 500 μl of phosphate-buffered saline, and 25 μl of the suspension was added to each well of a two-well slide. The smear was air dried, flame fixed, and heated at 80°C for 2 h.

At the time of detection of mycobacterial growth in the MGIT cultures, the MGIT tubes were vortexed for 10 to 15 s, and 4 ml of the cultures was centrifuged for 15 min at 3,000 × *g*. The supernatant was removed, the sediment was resuspended in 200 μl of phosphate-buffered saline, and 25 μl of the suspension was added to each well of a two-well slide. The smear was air dried, flame fixed, and heated at 80°C for 2 h.

FISH. For FISH, smears were hybridized according to the manufacturer's instructions. Briefly, prior to hybridization, the slides were immersed in 80% (vol/vol) ethanol for 15 min and allowed to air dry. One drop, approximately 25 μl, of the MTB probe was added to one of the smears, and 1 drop of the NTM probe was added to the other. Coverslips were added, and the slides were placed in a humidity chamber and incubated at 55°C for 1.5 h, followed by a posthybridization wash with prewarmed stringent wash solution at 55°C for 30 min. Subsequently, the slides were immersed in distilled water for 30 s and air dried. Finally, 1 drop of mounting medium was added to each of the two wells, coverslips were added, and the slides were incubated for 30 min at 55°C.

The hybridized smears were examined by fluorescence microscopy with a Zeiss Axiolab fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a 50-W mercury light source, a fluorescein isothiocyanate (FITC)-Texas red double filter, and a 100× oil immersion objective. Mycobacteria were identified on the basis of bright green fluorescence and morphology. The intensity of the fluorescence varied, depending on the species, the growth medium, and the age of the culture.

For a sample to be positive, one of the MTB and the NTM probes must be positive and the other must be negative. Double-positive and double-negative samples (positive and negative with both probes, respectively) were considered inconclusive, and the tests were repeated. Double-positive samples may indicate double infection and should be confirmed by other methods. Double-negative samples were stained by the Kinyoun staining method to determine the presence of any AFB on the slides.

Controls. Smears of *M. tuberculosis* H37Ra or H37Rv and *M. kansasii* ATCC 12478 grown on LJ medium were included in every assay as controls.

Identification of mycobacteria. Niacin strips (Difco) and the AccuProbe MTC test (Gen-Probe; San Diego, Calif.) were used for the identification of *M. tuberculosis* according to the instructions of the manufacturers. The following biochemical tests were used for species identification of *M. fortuitum*: arylsulfatase, iron uptake, nitrate reduction, sodium chloride tolerance, and urease.

RESULTS AND DISCUSSION

The specificities of the MTB and NTM probes were tested using selected clinically important *Mycobacterium* reference strains and isolates of *Pseudomonas*, *Streptococcus*, and *Staphylococcus* species identified as contaminants of mycobacterial cultures. The results shown in Table 1 are in agreement with the results of previous studies showing excellent specificity of the PNA probes (27). The MTB probe did not hybridize to any of the NTM species or any of the other bacterial species, and the NTM probe did not cross-hybridize to the MTBC species or the other bacterial species. Further, the study showed that the NTM probe did not hybridize to *M. fortuitum*.

The results of the test of the DAKO Probe MTB Culture Confirmation Test with samples from LJ cultures are shown in Table 2. Of the 53 AFB-positive cultures, 44 samples were found positive with the MTB probe and negative with the NTM probe and were thus identified as MTBC species. Four samples were found positive with the NTM probe and negative with the MTB probe and were thus identified as NTM species. These results were all in agreement with the results obtained by the reference methods. Four samples were double negative, and the result thus was inconclusive. For one of these samples, the AccuProbe MTC test was positive. Subsequent acid-fast staining showed that this sample contained only a few acid-fast-stain-positive bacteria (≤1+ staining). The remaining three samples all contained many acid-fast-stain-positive bacteria and were found negative by both reference methods. Subsequent biochemical testing identified the bacteria as *M. fortuitum*.

One sample tested double positive, and the AccuProbe MTC test and the niacin strip test results were also positive. Interestingly, the morphologies of the bacteria hybridizing with the MTB and NTM probes were markedly different: the bacteria positive with the MTB probe showed cord formation, whereas the bacteria positive with the NTM probe were independently located and were small. These observations strongly indicate the presence of two different species (13, 18). Furthermore, growth on LJ medium showed a mixture of colonies with rough and rather smooth surfaces, respectively, also indicative of two different species. Attempts to isolate and identify to the

TABLE 1. Results of the PNA probe-based FISH assay for mycobacterial reference strains and contaminants of mycobacterial cultures

Organism	Result[a] obtained with:	
	MTB probe-FITC	NTM probe-FITC
M. tuberculosis	+	−
M. bovis	+	−
M. avium	−	+
M. intracellulare	−	+
M. scrofulaceum	−	+
M. gordonae	−	+
M. kansasii	−	+
M. chelonae	−	−
M. fortuitum	−	−
M. xenopi	−	−
Pseudomonas spp.	−	−
Streptococcus spp.	−	−
Staphylococcus spp.	−	−

[a] +, positive; −, negative.

TABLE 2. Results for 53 AFB-positive cultures from LJ medium analyzed by the PNA probe-based FISH assay, the niacin strip test, and the AccuProbe MTC test

AccuProbe MTC and/or niacin strip test result	No. of positive results obtained in the PNA probe-based FISH assay with the following probe:		No. of cultures not identified by FISH
	MTB	NTM	
MTBC	44	0	2[a]
NTM	0	4	3[b]

[a] One was double negative; subsequent Kinyoun staining revealed ≤1+ staining for AFB. One was double positive; the organism morphologies were different.
[b] *M. fortuitum*, which does not hybridize with the NTM probe.

TABLE 3. Results for 77 AFB-positive cultures from MGIT medium analyzed by the PNA probe-based FISH assay, the niacin strip test, and the AccuProbe MTC test (discrepancy analysis)

AccuProbe MTC and/or niacin strip test result	No. of positive results obtained in the PNA probe-based FISH assay with the following probe:		No. of cultures not identified by FISH
	MTB	NTM	
MTBC	68	0	1[a]
NTM	0	5	0
Not identified	1[b]	1[b]	1[b]

[a] Subsequent Kinyoun staining revealed ≤1+ staining for AFB.
[b] Subsequent Kinyoun staining revealed ≤1+ staining for AFB and bacterial contamination.

species level the mixed colonies did not succeed and revealed only *M. tuberculosis*. Because the result was inconclusive, it was not included in the calculation of diagnostic sensitivity and specificity.

Of the 77 AFB-positive MGIT cultures, 68 samples tested positive with the MTB probe and 5 samples tested positive with the NTM probe (Table 3). Neither of the samples showed any hybridization with the other probe, and there was full correspondence with the results of the reference methods. Two samples were found double negative with both probes, and both had a low content of AFB. One of the samples was found positive by both the AccuProbe MTC test and the niacin strip test, whereas the other was found negative by both methods. The latter sample was contaminated and could not be further resolved.

The last two samples were found positive with the MTB probe and the NTM probe, respectively, but negative by both reference methods. Due to contamination with other bacteria, it was not possible to further resolve these samples.

The diagnostic sensitivities of the MTB probe were 98% for the LJ study and 99% for the MGIT study, whereas the diagnostic sensitivities of the NTM probe were 57% for the LJ study and 100% for the MGIT study. The specificity of both probes in both studies was 100%, a result which further supports the notion that PNA probes have a very high specificity.

These data show that the DAKO Probe MTB Culture Confirmation Test is well suited for the identification of MTBC and differentiation between tuberculous and nontuberculous *Mycobacterium* species in LJ and MGIT cultures. For both cultures, the MTB probe had a very high sensitivity. The sensitivity of the NTM probe was rather low in the LJ studies. That result was, however, due to a high relative recovery rate for *M. fortuitum* during that period of testing.

The DAKO Probe MTB Culture Confirmation Test has recently been evaluated with smears from other culture media. Drobniewski et al. (7) found in a study with a total of 74 cultures from MB/BacT medium sensitivities for the MTB and NTM probes of 100 and 82%, respectively. Both probes had a specificity of 100%, and in that study, the PNA test also identified a double-positive culture. Padilla et al. (22) evaluated the performance of the test with 129 smear-positive clinical specimens grown with BACTEC 12B as well as with MB/BacT. The sensitivities of the MTB probe were 88 and 100% for smears from BACTEC 12B and MB/BacT cultures, respectively, whereas the sensitivity of the NTM probe was 94% for smears from both cultures. Again, the specificity of both probes was 100%.

The sensitivity of the MTB probe obtained in our study compares well with that found in the other evaluations. The only sensitivity below 95% was found for cultures from the BACTEC 12B medium (22). This result may be related to the amount of bacteria present at the time of detection, which is substantially larger in a positive MB/BacT bottle than in positive BACTEC 12B and MGIT bottles. In the studies of Drobniewski et al. (7) and Padilla et al. (22), 1 ml of culture was used for sample processing. In preliminary investigations with 1-ml samples from MGIT cultures, we found a sensitivity below 95% (data not shown); this result led to an increase of the sample volume to 4 ml, which was used throughout this study. The increase in sample volume from 1 to 4 ml did not have any drawbacks.

In general, the sensitivity of the NTM probe is below 100%. This result is due to the fact that the NTM probe does not target all NTM species as, for example, *M. fortuitum*, as seen in this study. Other NTM species that are not targeted by the NTM probe are *M. flavescens*, *M. marinum*, *M. peregrinum*, *M. vaccae*, and *M. xenopi* (22, 27). If the species that are not targeted by the NTM probe are excluded from the calculations, the sensitivities of the NTM probe increase to 100% in our evaluation and to 96 and 100% in the studies of Drobniewski et al. (7) and Padilla et al. (22), respectively.

The DAKO Probe MTB Culture Confirmation Test is simple and easy to perform and requires only an incubator and a fluorescence microscope, which needs to be equipped with an FITC-Texas red double filter. A standard FITC filter cannot be used, as the autofluorescence of the mycobacteria makes it impossible to distinguish a positive result from a negative result. Unlike other molecular methods, the test has the advantage of allowing morphology to be used as an additional confirmation tool. Furthermore, as both an MTB probe and an NTM probe are used in the same test, the test provides an internal control and allows for the detection of mixed cultures of MTBC and NTM, as seen in this study and in the study of Drobniewski et al. (7). Moreover, the DAKO Probe MTB Culture Confirmation Test is superior to the niacin test for the detection of contaminated cultures. One disadvantage of the test is that the number of organisms needed for a positive result is relatively high. Furthermore, as mentioned above, the NTM probe does not target all clinically relevant *Mycobacterium* species; this fact may prove to be a problem in regions where one or more of these species are highly prevalent.

In conclusion, this new molecular technology based on PNA probes is simple, rapid, and well suited to the identification of MTBC and differentiation between MTBC and NTM and is easily adaptable to any mycobacterial laboratory. For the future, it would be interesting to have additional species-specific probes for the clinically important NTM species, such as *M. avium*, *M. intracellulare*, *M. kansasii*, and *M. xenopi*. Moreover, adaptation of the test to direct identification of *Mycobacterium* species in sputum specimens could prove highly valuable.

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